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The Effect of Phenylalanine Derivatives on the Solubility of Deoxyhemoglobin S

A Model Class of Gelation Inhibitors

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SUMMARY

Among the class of non-covalent inhibitors of deoxyhemoglobin S gelation, the aromatic amino acids have been shown to be the most effective. We have examined several synthetic chemical modifications of phenylalanine in order to determine the stereospecific constraints for inhibition of gelation by this class of compounds. The phenylalanine derivatives with ring modification by electron-donating groups (NH_2, CH_3, or OH) inhibited gelation to the same order of magnitude as phenylalanine (10–20% increase in deoxyhemoglobin S solubility at 32 mM). The phenylalanine derivative with the electron-withdrawing group NO_2 in the *p*-position behaved similarly, but the inhibitory effect was eliminated by NO_2 in the *m*- and possibly *o*-positions. Furthermore, side-chain modifications also eliminated the inhibitory effect. These studies, in conjunction with crystallographic analyses of the binding sites of gelation inhibitors, may provide a rational strategy for finding suitable compounds (whether covalent or non-covalent inhibitors) with appropriate physicochemical and biological properties to pursue as potential therapies with sickle cell disease.

INTRODUCTION

During the last decade, the biophysical characterization of deoxyhemoglobin S gelation has led to the investigation of numerous covalent and non-covalent inhibitors of gelation (1). These efforts have been encouraged by detailed analysis of the gel structure using electron microscopy (2, 3) and of the deoxyhemoglobin S crystal using X-ray crystallography (4, 5). Amino acids and small peptides have been studied as non-covalent inhibitors of gelation (6–10). Many did not significantly affect gelation, and several (e.g., aspartic acid, glutamic acid) even enhanced gelation, decreasing deoxyhemoglobin S solubility. However, of the noncovalent inhibitors, phenylalanine and tyrosine were among the most effective in increasing deoxyhemoglobin S solubility (6, 7).

The nature of the interaction between hemoglobin S and these noncovalent inhibitors is unclear, and there is no stereospecific requirement for either the D- or L-isomer (7, 10). The hydrophobic nature of the aromatic ring is clearly important, and the inhibitory effect of phenylalanine and tyrosine takes on additional significance in view of the excluded volume effect in neutralizing the inhibitory potential suggested by studies of oligopeptides from the β^6 region (7) and of $(Lys)_n$ (8); that is, rather than increasing or not affecting deoxyhemoglobin S solubility, these oligopeptides and polylysine decrease deoxyhemoglobin S solubility, presumably by taking up free water and reducing the amount of solvent accessible to hemoglobin. Furthermore, the non-ideal behavior of hemoglobin at the concentrations required for gelation (for example, at physiological concentrations of ~34 g/dl, the activity of hemoglobin is about 50 times its concentration) may further affect any interaction between these non-covalent inhibitors and hemoglobin S.

Derivatives of phenylalanine have been studied (7, 10–12) in an attempt to design a non-covalent modifier of gelation significantly more effective than phenylalanine or tryptophan and to gain additional understanding about the mechanism of action of these inhibitors of gelation. We now report the effect of several synthetic ring-substituted and other phenylalanine derivatives on gelation of deoxyhemoglobin S.

METHODS

All reagents were obtained from Aldrich Chemical Company (Milwaukee, Wisc.) and were of the highest available purity. The solvents were distilled prior to use. Amino acids (Fig. 1) *a*, *e*, *f*, and *g* were synthesized by

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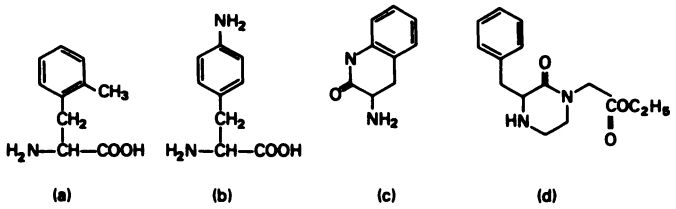
INHIBITORS:



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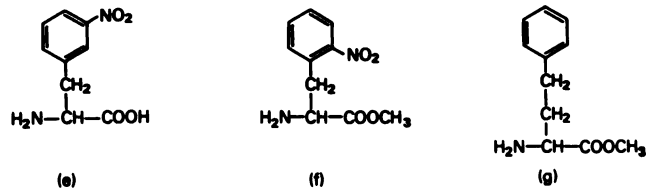

FIG. 1. *Chemical structure of the synthetic phenylalanine analogues*

The compounds which inhibit deoxyhemoglobin S gelation and increase its solubility are grouped as inhibitors (*a–d*) and include *o*-methylphenylalanine (*a*), *p*-aminophenylalanine (*b*), 3-amino-3,4-dihydrocarbostyril (*c*), and a piperazinone analogue (*d*). The compounds which did not affect deoxyhemoglobin gelation are grouped as noninhibitors (*e–g*) and include *m*-nitrophenylalanine (*e*), *o*-nitrophenylalanine methyl ester (*f*), and phenylethylglycine methyl ester (*g*).

condensation of the appropriately substituted benzyl chlorides with ethyl acetamidocyanoacetate in the presence of sodium ethoxide and subsequent hydrolysis with 6 N HCl (13). In the case of Compounds *f* and *g*, the methyl esters were obtained by treatment of the free acids with thionyl chloride in methanol. Nitration of phenylalanine yielded *p*-nitrophenylalanine, which was reduced to Compound *b* by catalytic hydrogenation (Pd/C). 3-Amino-3,4-dihydrocarbostyril (*c*) was prepared by catalytic hydrogenation of compound *f* and cyclization in acidic medium (14). The synthesis of the piperazinone derivative of phenylalanine (*d*) is described elsewhere (15). All derivatives were isolated as hydrochlorides. Compounds *b* and *d* had an L-configuration, whereas Derivatives *a, c, e, f,* and *g* were obtained as D,L-racemates. Purity of the final products was ascertained by nuclear magnetic resonance, infrared, and mass spectral analyses. Previous studies had shown that the configuration at the α-carbon atom of phenylalanine has no effect on its inhibitory activity (7).

The effects of the phenylalanine derivatives on gelation were evaluated by using the sedimentation assays described by Hofrichter *et al.* (16). Hemolysate from blood of individuals homozygous for hemoglobin S [determined by electrophoresis (hemoglobins F and A_2 were each less than 2%)] was prepared, concentrated to about 30 g/dl by ultrafiltration and vacuum dialysis, and dialyzed into potassium phosphate buffer, 0.15 M and pH 7.4. Stock solutions of the phenylalanine derivatives were made in buffer, and the pH was adjusted using concentrated KOH or HCl when necessary. The 5-mm quartz sample tubes were filled and capped under a nitrogen atmosphere. Final deoxygenation was achieved by the addition of fresh sodium dithionite solution (0.05 M final concentration). The final hemoglobin concentration was 24 g/dl. The samples were mixed at 5° for 16 hr, gelled at 37°, and centrifuged at 100,000 × *g* at 37° for 3 hr. The supernatant was thoroughly mixed and the hemoglobin concentration was measured by CN-methemoglobin determination (17).

RESULTS AND DISCUSSION

On the basis of the X-ray crystallographic structure for deoxyhemoglobin S, it has been proposed that phenylalanine and its derivatives interact with the hemoglobin region containing β^{73}-Asp, β^{85}-Phe, and β^{88}-Leu, thus blocking the complementary site in the polymer for the β^{S6}-Val region (9). The study of phenylalanine derivatives can be used to help define the structural requirements for this class of inhibitors and to gain more specific information about its mode of action. The derivatives we have examined are illustrated in Fig. 1 and their effects on the solubility of deoxyhemoglobin S are shown in Fig. 2 and Table 1. Almost all of the non-covalent reagents tested have exhibited nearly linear increase in HbS solubility with additive concentration (6–9, 12). Presumably because of the very low association constants of the molecular interactions, saturation was not reached. Whether some of the results in Fig. 2 differ from this significantly is not clear and requires further study of the intrinsic solubilities of the additives and a wider range of experimental data.

The phenylalanine derivatives could be separated into the inhibitors (Fig. 1, *a–d*) which had the same order of magnitude increase on deoxyhemoglobin S solubility as phenylalanine (24% for phenylalanine and 10–20% for derivatives at 32 mM) (Fig. 2), and the noninhibitors (Fig. 1, *e–g*) which did not significantly alter deoxyhemoglobin S solubility (<3% even up to 100 mM for some) (Table 1). Of the ring-substituted compounds, the former group

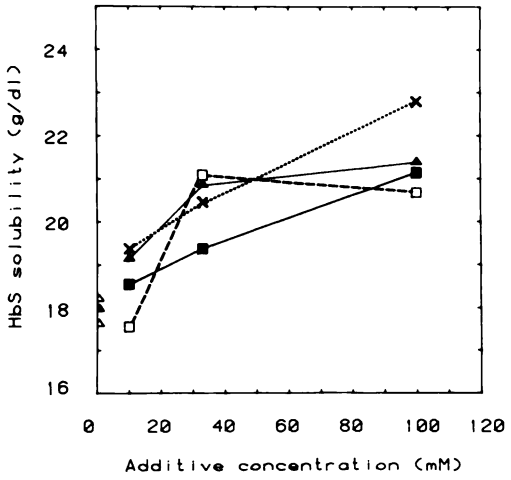

FIG. 2. *Increase in solubility of deoxyhemoglobin S by phenylalanine analogues*

The symbols represent the following compounds: ■,*o*-methylphenylalanine (Fig. 1, *a*); ▲, *p*-aminophenylalanine (Fig. 1, *b*); □, 3-amino-3,4-dihydrocarbostyril (Fig. 1, *c*); ×, a piperazinone analogue (Fig. 1, *d*).

TABLE 1

Effect of phenylalanine derivatives on gelation of deoxyhemoglobin S is manifest in deoxyhemoglobin S solubility

Phenylalanine at 32 mM increases solubility by 13% (which would correspond to 20.2 g/dl). The effect is approximately linear with phenylalanine concentration. Compounds *A–D* inhibit gelation, increasing deoxyhemoglobin S solubility.

Additive[a]	Concentration of additive	Deoxyhemoglobin S solubility[b]
	mM	*g/dl*
Control		18.2
		17.6
		17.9
a	10	18.1
	33	19.3
	100	21.1
b	10	19.1
	33	20.8
	100	21.3
c	10	17.5
	33	21
	100	20.6
d	10	19.3
	33	20.4
	100	22.7
e	10	18.3
	100	17.7
f	10	18
	33	17.5
g	10	18.3
	33	18.5
	100	18.4

[a] Additive letter corresponds to those of Fig. 1.

[b] The uncertainty in solubility determination is 1 g/dl, or 3%.

contains *o*-methyl phenylalanine (Fig. 1, *a*; Fig. 2) and *p*-aminophenylalanine (Fig. 1, *b*; Fig. 2) while the latter contains *m*-nitrophenylalanine (Fig. 1, *e*) and *o*-nitrophenylalanine methyl ester (Fig. 1, *f*).

The data suggest that ring modification by electron-donating groups such as NH_2 or CH_3 (or OH as in the case of tyrosine) in the *o*- or *p*-positions retains the inhibitor effect of the phenylalanine derivative (Fig. 1, *a* and *b*). The ring modification by the electron-accepting group, NO_2, appears to be much more complex. With NO_2 in the *m*-position (or the *o*-position with phenylalanine methyl ester), the inhibitory effect is eliminated (Fig. 1, *e* and *f*). However, with NO_2 in the *p*-position, the effect on deoxyhemoglobin S solubility is to increase it about 1.2 times that of unsubstituted phenylalanine (Fig. 2), as reported by Poillon and Kim (12). This suggests a possible steric interaction depending strongly on the position of NO_2. This marked dependence on the position of the substitution position of NO_2 and of fluorine reported by Poillon and Kim (12) indicate that the mechanism of gelation inhibition is not primarily mediated by hydrophobic interactions with the solvent.

The nature of the side chain is another important structural feature. Ross and Subramanian (9) have emphasized the importance of the distal relationship between the aromatic ring and the aliphatic side chain to which a hydrophilic group is attached. For example, the inhibitory effect is eliminated in homophenylalanine methyl ester (Fig. 1, *g*), which is similar to phenylalanine but contains an additional methylene group in the side chain. Further possibilities for ring modification are suggested by the fact that tryptophan exhibits a greater effect on inhibiting gelation than does phenylalanine (7). We found that 3-amino-3,4-dihydrocarbostyril (Fig. 1, *c*), a conformationally restricted derivative of phenylalanineamide, also inhibited gelation (Fig. 2), but not more than phenylalanine. The piperazinone derivative of phenylglycine (Fig. 1, *d*; Fig. 2) was also found to inhibit gelation. Perhaps this dipeptide structure provides additional sites for hydrogen bonding close to one of the intermolecular contact regions in the hemoglobin S gel. Nevertheless, its inhibitory effect remains of the same order of magnitude as that of phenylalanine. We also tested diphenylalanine and 2′-biphenylalanine. However, these compounds have limited solubility in water, and we observed no effect on solubility at the concentrations tested (<10 mM) (data not shown).

As a therapeutic agent, phenylalanine is limited by the large amounts required for sufficient increase in deoxyhemoglobin S solubility (about 30 mM for 24% increase) (18) relative to its potential toxicity (~2 mM) and its low membrane permeability.

The phenylalanine derivatives which inhibit gelation (Fig. 2), although they were not significantly more effective than phenylalanine, offer the advantage of greater solubility (~0.06 M for phenylalanine and >0.5 M for derivatives). Since observations can be made using higher additive concentration, it is not possible to study the specificity of binding by solution studies, such as nuclear magnetic resonance spectroscopy, and to locate the actual binding site or sites by X-ray crystallography. (The binding of several oligopeptides containing aromatic amino acids and their derivatives to hemoglobin crystals by difference Fourier analyses is now being examined by Drs. S. E. V. Phillips and M. Perutz, Medical Research Council Laboratory of Molecular Biology, Cambridge, England.) Such information would support the idea that this class of gelation inhibitors exhibits a specific interaction with the deoxyhemoglobin S molecule (7, 9) rather than inhibiting gelation primarily as a consequence of altering the solvent.

Progress has been made in searching for a non-covalent modifier of gelation of potential therapeutic value, with 5-bromotryptophan being the most effective inhibitor to date (12). However, the desired efficacy (which we estimate to be about 1–2 orders of magnitude greater than that of phenylalanine) has not been reached. Although other strategies for anti-sickling compounds—including covalent modification and decreasing mean corpuscular hemoglobin concentration (1, 19)—are of considerable potential, there are many attractive features to attempts to use physicochemical and structural data to design stereospecific inhibitors of intermolecular contacts. Studies of phenylalanine derivatives as a model class of compounds with which to study inhibitor binding and molecular interaction should give insight about the design of possible drug therapy of sickle cell anemia.

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